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Study of *Leishmania* antigens for the diagnosis of human visceral leishmaniasis

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“Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning”

Albert Einstein

Aos meus pais

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Abstract

Leishmaniasis is a worldwide disease caused by a digenetic protozoan parasite which can appear in different clinical forms, depending on the *Leishmania* species involved and the immune status of the host. It is the most important vector-borne disease after malaria and sleeping sickness.

The main reservoir for human visceral leishmaniasis is the dog, *Canis familiaris*. Infected domestic dogs serve as reservoirs of the disease for people in several areas where leishmaniasis is endemic. Both dogs and humans are infected via the bite of female phlebotomine sandflies, which breed in forest areas, caves, or the burrows of small rodents.

Leishmaniasis is a diagnostic challenge because of the wide spectrum of clinical manifestations that it may present. Visceral leishmaniasis is a fatal disease if untreated and the therapeutic success comes in line with an early detection of the disease. Thus, accurate diagnosis tests are needed, especially for the detection of asymptomatic patients. The perfect diagnosis methods should be easy to perform, not expensive and highly sensitive and specific. In this work, we explored ELISA based diagnosis tests for the accurate diagnosis of human VL.

Key words: human leishmaniasis, ELISA, diagnosis.

Resumo

A leishmaniose é uma doença do mundo, causada por um protozoário parasita que pode aparecer em diferentes formas clínicas, dependendo das espécies de *Leishmania* envolvidas e o estado imunológico do hospedeiro. É a mais importante doença transmitida por vetores após malária e a doença do sono.

O principal reservatório para a leishmaniose visceral humana é o cão, *Canis familiaris*. Cães domésticos infetados servem como reservatórios da doença para os humanos em diversas áreas onde a doença é endémica. Tanto os cães e como os humanos são infetados através da picada de flebotomíneos do sexo feminino.

A leishmaniose é um desafio a nível do diagnóstico, considerando o amplo espectro de manifestações clínicas que podem apresentar. A leishmaniose visceral é uma doença fatal se não tratada e o sucesso terapêutico depende da deteção precoce da doença. Assim, são necessários testes de diagnóstico precisos e exatos, especialmente para a deteção da doença em pacientes assintomáticos. O método de diagnóstico ideal deve ser fácil de executar, não ser caro e ser altamente sensível e específico. Neste trabalho, exploramos testes diagnósticos baseados no método de ELISA para o diagnóstico preciso da leishmaniose humana.

Palavras-chave: leishmaniose humana, ELISA, diagnóstico.

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Abbreviations

VL	Visceral Leishmaniasis
CVL	Canine Visceral Leishmaniasis
CL	Cutaneous Leishmaniasis
MCL	Mucocutaneous Leishmaniasis
PKDL	Post-kala azar Dermal Leishmaniasis
ZVL	Zoonotic Visceral Leishmaniasis
SLA	Soluble *Leishmania* Antigen
*Lic*TXNPx	*Leishmania infantum* cytosolic tryparedoxin peroxidase
rK39	Recombinant K39 antigen
rK28	Recombinant K28 antigen

I. Introduction

Leishmaniasis is a vector-borne disease caused by protozoa of the genus *Leishmania*, family Trypanosomatidae and order Kinetoplastida(2). It is a worldwide disease caused by a digenetic protozoan parasite which can appear in different clinical forms, depending on the *Leishmania* species involved and the immune status of the host. It is the most important vector-borne disease after malaria and sleeping sickness (3, 4). There are three major clinical types of this infection: cutaneous (CL), mucocutaneous (MCL) and visceral (VL). VL, also known as kala azar, is a severe infectious disease caused by *L. donovani* in East Africa and the Indian subcontinent and *L. infantum* in Latin America and the Mediterranean basin (5, 6) .

The main reservoir for human VL is the dog, *Canis familiaris*. Infected domestic dogs serve as reservoirs of the disease for people in several areas where leishmaniasis is endemic. Canine leishmaniasis (CVL) is also sometimes found in non-endemic countries because of international tourists and immigrants who bring infected pets and dog importation from endemic areas. Dogs can be asymptomatic carriers of leishmaniasis maintaining this status for a long period of time, being nevertheless highly competent to transmit the parasite to sandflies (7) . Both dogs and humans are infected via the bite of female phlebotomine sandflies, which breed in forest areas, caves, or the burrows of small rodents (4, 7, 8).

Leishmaniasis is a diagnostic challenge because of the wide spectrum of clinical manifestations that it may present. VL is a fatal disease if untreated and the therapeutic success comes in line with an early detection of the disease. Thus, accurate diagnosis tests are needed, especially for the detection of asymptomatic patients. The perfect diagnosis methods should be easy to perform, not expensive and highly sensitive and specific (3). In this work, we explored ELISA based diagnosis tests for the accurate diagnosis of human VL.

1.1 Transmission and life cycle

Leishmania parasites are transmitted to mammals by the bite of female phlebotomine sand flies and occasionally by the sharing of needles, by blood transfusion, or by congenital transmission.

There are two types of VL, which differ in their transmission characteristics: zoonotic VL is transmitted from animal to vector to human and anthroponotic VL is transmitted from human to vector to human. In the former, human are occasional hosts and animals, mainly dogs, are the reservoir of the parasite (3).

The life-cycle of *Leishmania* has two distinct forms: the flagellated promastigotes found in the gut of the arthropod vector and non-motile amastigotes, which develop intracellulary in the mammalian host. Promastigotes injected into the skin during sand fly bite are internalized by dendritic cells and macrophages in the dermis where they lose their flagella during the differentiation into the amastigote form. They multiply and survive within the phagolysosome through complex host-parasite interactions (9, 10). The incubation period can vary from weeks to months and during this period disease symptoms may gradually appear and worsen with disease manifestations ranging from self-healing skin lesions to diffuse cutaneous and mucosal manifestations and, in some cases, to severe visceral involvement to spleen, liver and lymph nodes depending on species of *Leishmania* and the immune status of the host *(9, 11)*.

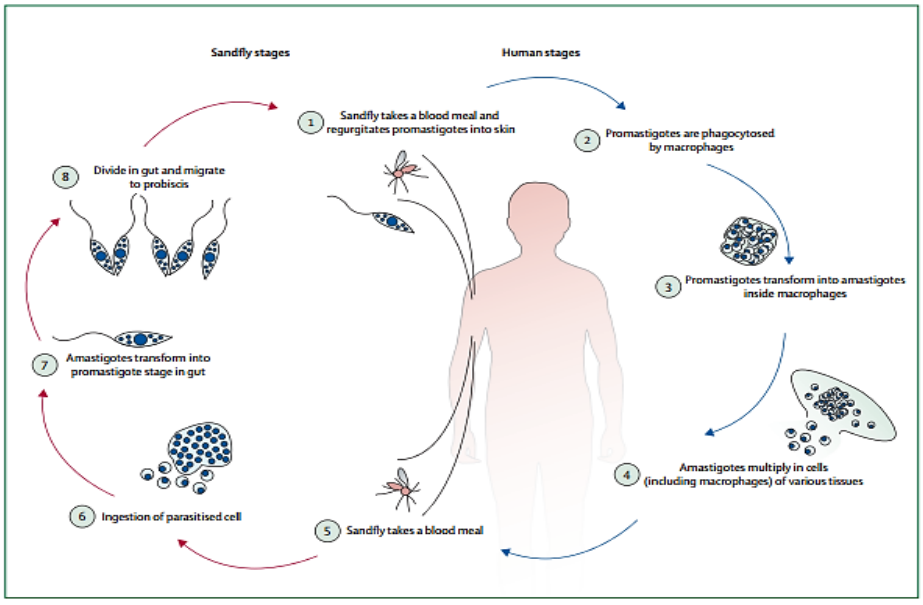

Fig. 1 Life cycle of *Leishmania* spp. parasites, *adapted from* (12)

1.2 Epidemiological data

The epidemiology of leishmaniasis depends on the parasite species involved, the local ecological characteristics of the transmission sites, the current and past exposure of the human population to the parasite and widely varying human behavior, such as socioeconomic factors, malnutrition, environmental changes, population movement and climate changes (4).

Leishmaniasis is one of the world's most neglected diseases affecting largely the poorest of the poor, mainly in developing countries. 350 million people are considered at risk of contracting leishmaniasis with an approximate yearly incidence of 1.5-2 million (3, 12).

Leishmania species are classified in two different groups: Old World (Mediterranean, Middle East and Asian countries) that includes *L. major, L. tropica, L. donovani* and *L. infantum*, and New World (Latin America) that in*cludes L. chagasi* (synonymous with *L. infantum*)*, L. mexicana, L. amazonensis, L. braziliensis* and *L. guyanensis (13).*

Leishmaniasis consists of four main clinical syndromes: cutaneous, mucocutaneous, visceral and post-kala-azar dermal leishmaniasis (PKDL). **Cutaneous leishmaniasis** (CL) is the most common form of leishmaniasis. CL can be sub-divided in Localized Cutaneous Leishmaniasis (LCL) and in Diffuse Cutaneous Leishmaniasis (DCL). About 95% of CL cases occur in the Americas, the Mediterranean basin, and the Middle East and Central Asia. Over two-third of CL new cases occur in six countries: Afghanistan, Algeria, Brazil, Colombia, Iran and Syria. An estimated 0.7 million to 1.3 million new cases occur worldwide annually although death cases are rare since CL is normally a self-healing condition (14, 15).

Mucocutaneous leishmaniasis leads to partial or total destruction of mucous membranes of the nose, mouth and throat. Almost 90% of mucocutaneous leishmaniasis cases occurs in the Plurinational State of Bolivia, Brazil and Peru (14, 15).

Visceral leishmaniasis (VL also known as kala-azar) is fatal if left untreated. It is characterized by irregular bouts of fever, weight loss, enlargement of the spleen and liver and anemia. It is highly endemic in the Indian subcontinent and in East Africa. An estimated 200 000 to 400 000 new cases of VL occur worldwide each year. Over 90%

of new cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan (7, 14).

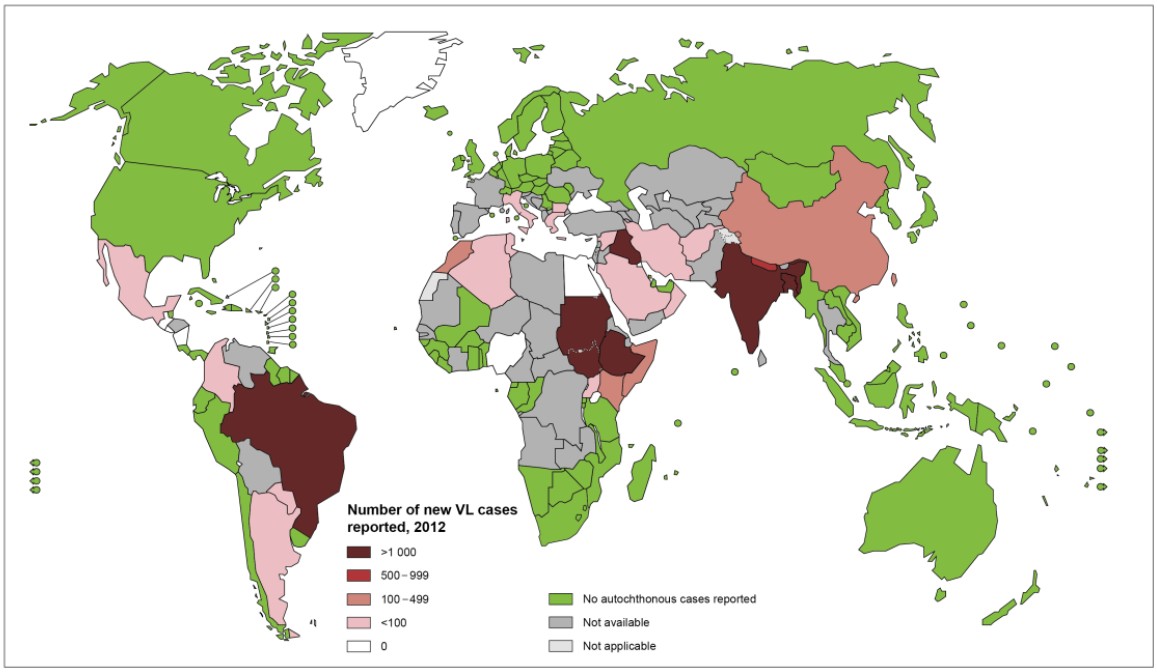

Fig. 2 Status of worldwide endemicity of visceral leishmaniasis, 2012, *adapted from* (16)

In populations where substantial levels of transmission have been sustained over periods of years, in endemic areas, a large proportion of the adult population acquired immunity to the parasite. Naïve or immunosuppressed adults who enter an endemic area are nevertheless at risk of the disease (4).

Since the early 1990s, the incidence of human leishmaniasis has displayed a large increase in Mediterranean countries of southern Europe mainly under the influence of human immunodeficiency virus (HIV) infection. In some regions the officially reported cases almost doubled between 1997 and 2004, and 25 to 70% of adults with VL are co-infected with HIV. Further, leishmaniasis is a major public health problem in the eastern mediterranean region where cutaneous and visceral leishmaniasis are both endemic (15).

In Portugal, the Alto Douro region has the highest incidence with 8.3 human cases/100000 inhabitants and 12.54% seroprevalence in dogs (14, 17, 18). The metropolitan Lisbon region and Algarve have the higher HIV/*Leishmania* co-infection case numbers with 0.2/100000 and 1.2 cases/100000 inhabitants, respectively, but very few recent cases among immunocompetent subjects (14, 17, 18).

1.3 Clinical presentation

Leishmaniasis is characterized by a spectrum of clinical manifestations: ulcerative skin lesions developing at the site of the sandfly bite (localized cutaneous leishmaniasis, LCL); multiple non-ulcerative nodules (diffuse cutaneous leishmaniasis, DCL); destructive mucosal inflammation (mucosal leishmaniasis); and disseminated visceral infection (visceral leishmaniasis) that can lead to post-kala azar dermal leishmaniasis (PKDL)(12).

People can carry some species of *Leishmania* asymptomatically for long periods, without becoming ill. In humans, the reported incubation period for cutaneous leishmaniasis can be as short as 1-2 weeks or as long as several months when it is caused by New World species, and up to three years when Old World species are involved. The incubation period for visceral leishmaniasis is 10 days to several years; most cases seem to become apparent in two to six months (19, 20).

1.3.1 Cutaneous Leishmaniasis

CL usually produces ulcers on the exposed parts of the body, such as the face, arms and legs (Fig.3). There may be a large number of lesions – sometimes up to 200 – which can cause serious disability. When the ulcers heal, they invariably leave permanent scars, which are often the cause of serious social prejudice (21). Of note, leishmaniasis caused by visceral species seldom cause cutaneous disease (18, 22).

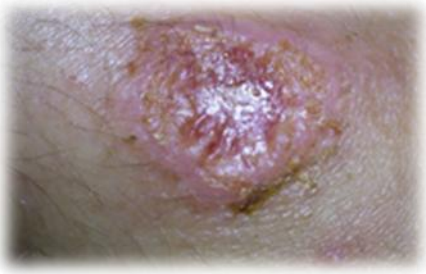

Fig. 3 Cutaneous leishmaniasis, *adapted from* (21).

1.3.2 Mucocutaneous Leishmaniasis

In mucocutaneous leishmaniasis, the lesions can lead to partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues (Fig.4). This disabling form of leishmaniasis can lead to the sufferer being rejected by the community (23).

Fig. 4 Mucocutaneous Leishmaniasis, *adapted from* (12)

The clinical spectrum observed in patients indicates the complexity of leishmaniasis several *Leishmania spp* can cause disease (table 1), and many sandfly and mammalian species have been implicated as vectors and reservoir hosts, respectively (12).

Table 1 Species of *Leishmania* that cause human disease, *adapted from* (12)

	Main Clinical Pathology	Transmission cycle	Main geographical distribution
New World *Leishmania spp*.			
L. brazilliensis	LCL, mucosal	Zoonotic	South America, parts of Central America, Mexico
L. garnhami	LCL	Zoonotic	South America
Old World *Leishmania spp*.			
L. major	LCL	Zoonotic	Central Asia, north Africa, middle east, East Africa
L. donovani	Visceral, LCL	Anthroponotic	Africa, central Asia, southeast Asia
Old and New World *Leishmania spp*.			
L. infantum =(L.chagasi)(24)	Visceral, LCL	Zoonotic	Europe, north Africa, Central America, South America

1.3.3. Visceral Leishmaniasis (VL)

VL is usually an insidious, chronic disease among the inhabitants of endemic areas; however, the onset may be acute in travelers from *Leishmania*-free areas (Fig.5). In some cases (especially in Africa), a primary granuloma appears on the skin at the site of the bite before the systemic signs (15, 20).

The most common symptoms of visceral leishmaniasis are a prolonged undulant fever, weight loss, decreased appetite, signs of anemia, and abdominal distension with splenomegaly and hepatomegaly. Thrombocytopenia may cause bleeding tendencies, including petechiae or hemorrhages on the mucous membranes, and leukopenia can result in increased susceptibility to other infections (20).

Other symptoms may include coughing, chronic diarrhea, darkening of the skin, lymphadenopathy, and in many cases, signs of chronic kidney disease. Mild cases with only a few symptoms may resolve spontaneously. Unless VL is treated, the majority of the cases are eventually fatal, often from secondary infections and other complications. Fulminant disease or atypical cases can also occur, especially in patients co-infected with HIV. People with successfully treated infections continue to carry the parasite, and the disease may recur if they become immunosuppressed. Similarly, asymptomatically infected individuals may later develop clinical signs (12, 19, 20).

Fig. 5 Visceral leishmaniasis. *adapted from* (14, 25).

Post-kala azar dermal leishmaniasis (PKDL) occurs after recovery in some cases of visceral leishmaniasis caused by *L. donovani* (Fig.6). This syndrome is characterized by a maculopapular, macular or-nodular rash around the mouth, which spreads. In Africa, PKLD is common, usually occurs within 6 months of visceral leishmaniasis, and typically disappears within a year without treatment. In South Asia, this syndrome is relatively rare, occurs several years after VL has been cured, and required prolonged treatment. In India, PKLD is seen in 1-3% of successfully treated cases of visceral leishmaniasis (20).

Fig. 6 Post-Kala Azar dermal leishmaniasis, adapted from*(25)*.

1.4 Reservoir

The transmission of *L. infantum* from domestic dogs by the bite of infected sandflies was first demonstrated in the 1930s (26, 27). Subsequently, many studies have confirmed the role of the domestic dog as the primary reservoir of Zoonotic Visceral Leishmaniasis (ZVL): dogs often have long-lasting infections with a high prevalence of both infection and infectiousness, being common in the peridomestic environment in which most ZVL transmission occurs (11). In infected dogs the disease can present symptoms (approximately 45.4%) (fig.7) or not (24.4%), with the latter being more common (28).

There are many *Leishmania* reservoirs all over the world. In the Americas the most common reservoirs are sloths, opossums, small forest rodents such as the hydrax and peri-domestic dogs (20). In India, the asymptomatic and symptomatic individuals serve as a reservoir for the parasite. This is a particularly case of an anthroponotic life cycle where only 1 in 7 infected with *L. donovani complex* become symptomatic (20).

Fig. 7 A Dog with visceral leishmaniasis, *adapted from* (29).

II. Diagnostic methods

The accurate diagnostic of *Leishmania* infection is a huge challenge because of the wide spectrum of clinical manifestations that it may be presented (15).

Early diagnosis is essential for both individual patients and for the community. Adult patients in Sudan with severe anemia, malnutrition and long duration of illness were shown to be at an increased risk of death (30). Untreated VL patients act as a reservoir for parasites and therefore contribute to disease transmission in anthroponotic VL areas. Early case-finding and treatment is therefore considered an essential component of VL control (31-33). The concomitant detection and treatment of PKDL patients is also likely to be beneficial and the feasibility, impact and cost of a PKDL management strategy should therefore be properly evaluated (3).

As the clinical presentation of VL lacks specificity, confirmatory tests are required to decide which patients should be treated. Such tests should be highly sensitive (>95%) as VL is a fatal condition, but also highly specific due to the non-pathognomonic symptomatology assuring the proper use of anti-*Leishmania* drugs. Ideally, a test should be able to make the distinction between acute disease and asymptomatic infection, because none of the drugs currently available is safe enough to treat and follow asymptomatic infections. Moreover, such tests should be simple and affordable (3).

The direct methods include parasite detection and indirect methods can be divided in molecular (Polymerase Chain Reaction (PCR) and quantitative Polymerase Chain Reaction (qPCR)) and serological (Direct Agglutination Test (DAT), Indirect Fluorescent Antibody Test (IFAT), Enzyme Linked Immunosorbent Assay (ELISA), Flow Cytometry and nanodiagnostic).

However, we have non-*Leishmania* tests that evaluate serological and physiological parameters indicatives of *Leishmania* infection. A reduction in the number of erythrocytes, leucocytes and platelets (pancytopenia) was found to be highly specific (98%) for VL in suspected clinical patients in Nepal but with low sensitivity (16%) (34). Marked polyclonal hypergammaglobulinemia (the production of high titers of non-specific antibody), a common finding in VL, can be detected by a formol gel test (FGT; also called the aldehyde test), which is still used in East Africa and Asia because of its simplicity and low cost. However, as the sensitivity of this test is poor (as low as 34%), and its use has been recommended to be discontinued (35, 36).

2.1 Direct methods

2.1.1 Parasite detection

The visualization of the amastigote form of the parasite by microscopic examination of aspirates from lymph nodes, bone marrow (fig.8) or spleen is the classical confirmatory test for VL. Although the specificity is high, the sensitivity of microscopy analysis varies, being higher for spleen (93-99%) than for bone marrow (53-86%) or lymph node (53-65%) aspirates (37-41).

However, spleen aspiration can be complicated by life threatening hemorrhages in ~0.1% of individuals and therefore requires considerable technical expertise (7, 42), as well as facilities for nursing surveillance, blood transfusion and surgery. Moreover, the accuracy of microscopic examination is influenced by the ability of the laboratory technician and the quality of the reagents used. The detection of parasites in the blood or organs by culture or by using molecular techniques such as PCR is more sensitive than microscopic examination but these techniques remain restricted to referral hospitals and research centers, despite efforts to simplify them (3, 36, 43).

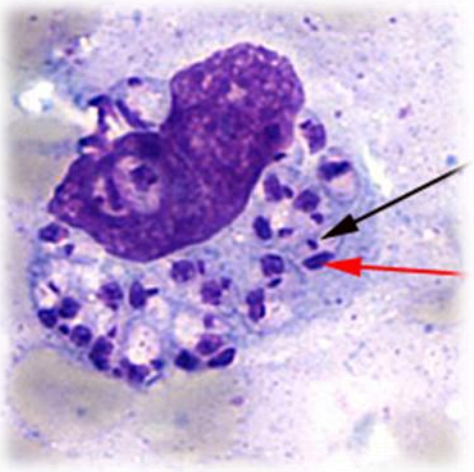

Fig. 8 Light-microscopic examination of a stained bone marrow specimen from a patient with visceral leishmaniasis—showing a macrophage containing multiple *Leishmania* amastigotes (the tissue stage of the parasite). Note that each amastigote has a nucleus (red arrow) and a rod-shaped kinetoplast (black arrow). Visualization of the kinetoplast is important for diagnostic purposes, to be confident the patient has leishmaniasis, *adapted from* (1)

2.2 Indirect methods

2.2.1 Molecular methods

Molecular parasitological diagnoses have been extensively explored during the past decade. It is essentially performed by PCR based methods and it is particularly useful in cases with low parasite burden (12). The detection of *Leishmania* parasites by PCR methods is highly specific and sensitive, with values reaching up 100% (44). However, these techniques remain complex and expensive, and in most VL-endemic countries are therefore restricted to a few teaching hospitals and research centers (5).

Molecular approaches have shown great potential for the diagnosis of leishmaniasis, follow-up after treatment, disease control and epidemiology.

2.2.1.1 PCR (Polymerase Chain Reaction)

In the last decade, PCR has proved to be a sensitive, specific and powerful tool to detect *Leishmania* DNA. PCR-based assays currently constitute the main molecular diagnostic approach of researchers and health professionals (43, 45, 46).

PCR for amplification of defined parasite DNA sequences is highly sensitive for animals with clinical disease. The sensitivity of a PCR assay primarily depends upon three factors: the physicochemical conditions of the reaction, the concentration and nature of the DNA target, and the selected PCR primers (47-49).

Leishmania kinetoplast DNA contains abundant, parasite-specific, repetitive sequences (organized in thousands of maxicircles and minicircles) that are very useful as targets for nucleic acid-based detection. The PCR assay based on kinetoplast amplification is probably the most sensitive because this molecular target is present in about 10 000 copies per parasite. Conserved regions of *Leishmania* small-subunit ribosomal RNA (SSU rRNA) are also used as PCR target (46, 50, 51).

In comparison with direct methods, PCR shows more sensitivity in confirming asymptomatic infections, which are characterized by low or intermittent parasite. Thus, PCR-based methods allow a highly sensitive and specific detection of *Leishmania* parasite even in samples where parasite load is low (43, 52).

The number of positive diagnoses would increase due to parasite detection in patients whose smears are negative, and adequate treatment can be applied as early as possible (53). PCR targets should be chosen according to the aim to which it is directed. PCR-based assays using kinetoplast DNA targets are the most sensitive for the diagnosis of leishmaniasis, but they identify *Leishmania* parasites only to the generic and/or subgeneric level, whereas other PCR targeting intergenic regions in nuclear DNA are better when rapid and reliable species identification is needed (51). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) allowed identification of parasites at the complex level.

However, the technical complexity and access to sophisticated equipment may reduce the practicality of the method in developing countries, which in turn are the most affected by VL (46, 54). A rapid dipstick test has been developed to overpass these limitations named *Leishmania* OligoC-Test. This test is simplified and it is based on the amplification of a small sequence of the 18S rRNA gene, followed by visualization of the PCR products on a dipstick by hybridization with a gold-conjugated probe. There is no need of expensive equipment or high technology, and so, the OligoC-test can be conducted in low-level-equipped laboratories and present a low-cost for the users. This is a very important issue in high endemicity locations, where poor population has a limited access to appropriate health care (46). This test presents a sensitivity of 96.4% and a specificity of 88.8% (55).

2.2.1.2 qPCR (quantitative Real-Time Polymerase chain reaction)

This technology has revolutionized the way clinical microbiology laboratories diagnose human pathogens, by adding reliability and speed. The combination of excellent sensitivity and specificity, low contamination risk and ease of performance has made qPCR technology an appealing alternative to other diagnostic methods (49, 56).

qPCR can be used to assess host tissue quantification of parasites, which is useful in monitoring disease progression. Absolute quantification is determined by comparison to standard curves from parasite DNA. When sensitivity must be maximized, namely when detection and quantification are needed, high-copy number targets (rRNA genes and kinetoplast DNA minicircles) should be chosen the best target. The parasite viability can also be demonstrated by qPCR using RNA instead of DNA (43, 50, 52).

Real-time qPCR would become the reference technique in the future because it is more rapid and less prone to contamination (no post-PCR handling is required), although, when compared with conventional PCR, the cost can be 3 fold higher (56).

As the choice of the PCR assay will depend on the aim of the test, the choice of the target, evaluation of the cost/benefit has to be done (51).

2.2.2 Serological diagnosis

The use of antigens for the serodiagnosis of tropical diseases is often limited by the difficulty in producing large quantities of the antigen (7, 57). However, serodiagnosis is easy to perform, cheap to make and has both high specificity and sensibility depending on the antigen (57).

2.2.2.1 DAT (Direct Agglutination Test)

In 1985, El Harith *et al.* developed a DAT for VL with high sensitivity and specificity (58). This test is semi-quantitative and uses microtitre plates with V-shaped wells in which increasing dilutions of serum or blood eluted from filter paper are mixed with stained killed *L. donovani* promastigotes.

Since 1986, the DAT has been extensively validated in most VL-endemic areas. Thirty studies were included in a recent meta-analysis, showing sensitivity and specificity estimates of 94.8% (95% confidence intervals (CI), 92.7-96.4) and 97.1% (95% CI, 93.9-98.7), respectively (59). The DAT performance was neither dependent on the region nor on the *Leishmania* species. DAT antigen production was initiated in some endemic countries but the production could not always be sustained, and quality control remained an issue. The cost of the antigen is in the range of 1-2EUR/test. Although highly sensitive and specific, the DAT requires substantial manipulation, and can only be read after a minimum of 8 hours incubation (5). Moreover, a multi-center study reported low reproducibility owing to problems reading the results and the heat- and shock sensitivity of the liquid antigen (60).

2.2.2.2 ELISA (Enzyme linked immunosorbent assay)

Enzyme linked immunosorbent assay (ELISA) has been used in the serodiagnosis of VL. ELISA is the best choice for the development of a rapid and reliable diagnostic method, because it is more practical, easy to standardize and suitable for mass screening (6). However, sensitivity and specify of ELISA depends upon the antigen used (36).

Previously, the following proteins, HSP83, hsp70, RkLO8, rK26, rK9, SIR2, , *Lic*TXNPx, rK39 and rK28, have been individually studied and a combination named LAM (*Lic*TXNPx+rK39) was previously reported (6, 45). **Soluble *Leishmania* antigens** (SLA) are produced from the promastigote stage of L. *infantum*. SLA are the most commonly used antigens in ELISA. These ELISAs have demonstrated sensitivities and specificities of 80 to 100% and 85 to 95%, respectively (61). However, the use of SLA and occurrence of false positive results in cases of Chagas disease have been documented (62-66).The use of total soluble antigens is limited due to problems of reproducibility and manufacturing (45).

***Leishmania infantum* cytosolic tryparedoxin peroxidase** (*Lic*TXNPx) antigen, is a member of unique enzymatic *Leishmania* cascades for detoxification of peroxides (67), as a highly immunogenic probe during natural human or experimental canine infectious. ELISA test based on *Lic*TXNPx presented a sensitivity of 78.6% and a specificity of 89.0% in the detection of CVL (45).

Recombinant K39 antigen (rK39) is a 39-amino-acid repetitive immunodominant B-cell epitope of the 230kDa kinesin related protein of *L. chagasi* (LcKin*)*.(68, 69). ELISA test based on *Lic*TXNPx presented a sensitivity of 93.5% and a specificity of 92.38% (9). An **immunochromatographic strip test with recombinant k39 antigen** (rk39 ICT) was developed (70). The InBios Kalazar Detect rapid test utilizes the recombinant *L. chagasi* antigen rK39(69). It has previously been reported that *L. chagasi*, *L. donovani*, and *L. infantum* all contain the gene encoding the LcKin protein (69). A membrane strip containing a conjugate dye region is coated with this protein. Through capillary action, the patient serum will react with the dye and antigen to quickly indicate the presence of anti-rK39 immunoglobulin G (IgG) in a patient sample (70).

It is easy to use in the field and results are available after 15 minutes. The initial study showed 100% sensitivity and 98% specificity (71), but this particular format (Arista Biologicals, Allentown, PA, USA) is no longer commercially available because several

limitations (71). First, additional field testing of the impregnated strip in other parts of the world where kala-azar is endemic is needed to confirm the applicability of our findings. Second, firm diagnoses of other diseases were not reached in some of our strip-test negative patients. An evaluation in Sudan of ICT from the same producer showed only 67% of sensitivity (72). An ICT produced by a different company (INBIOS, Seattle, WA, USA) proved to be a good diagnostic guide in suspected VL cases in India(73) and in Bangladesh. Sarker *et al.* found excellent sensitivity (96.6%) and specificity (98.3%) with this ICT (74). In Nepal, an early prototype showed a specificity of only 71% in controls with clinical signs of VL (75); however, better specificity (above 90%) was obtained with later generations of the InBios ICT and with an ICT produced by DiaMed AG, Switzerland (34, 76).

Recombinant K28 antigen (rK28) is a fusion polyprotein comprising regions of *L. donovani* haspb1 (*L. infantum* k26 homologue), *L. donovani* kinesin (*L. infantum* k39 homologue) and *L. donovani* haspb2 (*L. infantum* K9 homologue) (7, 9). The ELISA tests based on these antigens have shown sensitivities of 88.4-99.6% and specificity 90.53-98.95% (9).

Rk28 ELISA is more sensitive and specific than rk39, and have a high predict diagnostic accuracy than rk39 (9).

The presence of **parasite antigens released in urine** can be linked to the level of renal physiology as well as status of infection present in the host. In addition, the urinary antigens may be also useful in diagnostic and prognostic purposes (77). The diagnostic capabilities of urinary antigens have been reported in various infectious diseases like schistosomiasis (78), malaria (79), Chagas disease and river blindness. The presence of soluble urinary *Leishmania* antigens in VL has been successfully reported in various studies (77, 80), however, their characterization has not been done in most of the cases (80). In the context to unavailability of a perfect diagnostic and prognostic tool, the urinary *Leishmania* antigens offer a better prospective (77).

Recently, in Sudan and other countries, a new latex agglutination test (KAtex; Kalon Biological, UK) for detecting *Leishmania* antigen in urine was evaluated in patients suspected to have VL(81). In urine, KAtex detects a low-molecular-weight glycoconjugate (5–20 kDa) that is present in the promastigote and amastigote stages of the parasite (82). Antigen detection in urine (ADU) by KAtex is quick and easy to perform. The use of urine over other conventional samples taken for VL diagnosis offers an advantage in that urine is easy to obtain; moreover, ADU is highly sensitive during clinical episodes. Therefore, ADU is appropriate for diagnosis of primary VL, for monitoring the efficacy of treatment,

and for detection of subclinical infection. From a clinical standpoint, a positive ADU result in the absence of symptoms is a marker of the parasite's presence but not necessarily of the disease (81). ADU presents a high sensitivity in the diagnosis of VL during primary episodes (42/49, 84.6%) and relapses (16/18, 88.8%) when the parasite load is high (81).

2.2.2.3 IFAT (Indirect Fluorescence Antibody Test)

Fluorescent antibody techniques are extremely valuable qualitative tolls but they do not provide quantitative data. IFAT is a semi-quantitative assay that allows evaluation of anti-*Leishmania* antibody titers produced by infected individuals (83, 84).

On the other hand, the interpretation of IFAT may be subjective, dependent on the skill of the operator, and although the specificity and sensitivity can be high (100% and 90% respectively), in respect to asymptomatic infections IFAT has sensitivity significant lower than rk39 ELISA test (83, 85).

2.2.2.4 Flow cytometry

The use of flow cytometry for the serodiagnosis of leishmaniasis was initiated by a study that evaluated the presence of antibodies against *L. brazilliensis* promastigotes (86). Subsequently, the same approach was adapted for determining anti-*L. infantum* antibody levels in serum samples from dogs with CVL (87). Recently, the applicability of flow cytometry serology as a novel assay for diagnosis of CVL was reported, and this method showed great sensitivity (95%) and specificity (100%) for CVL diagnosis (88, 89).

A new method was developed based on flow cytometric analysis since this technology is considered to be highly sensible and specific for the diagnosis of infectious diseases (90). This method combines antigen-coated magnetic microspheres, immunomagnetic separation and flow cytometry for the detection of specific antibodies to *Leishmania* (91).

This technique possesses several advantages for immunoassays such as high throughput capacity, possibility of analyze quantification, reduced sample volume, high reproducibility and sensitivity, a wide dynamic range, and, the most exciting to all, the potential for multiplexing (90).

2.2.2.5 Nanodiagnostic

Micro and nanotechnology have been applied in the development of biosensors that emerge as promising diagnostic methods (92). Microsphere-based immunoassay with covalent binding between an antigen or antibody to magnetic microspheres have been considered promising alternatives for serological analysis (93).

The use of nanotechnologies for diagnostic applications shows great promise to meet the rigorous demands of the clinical laboratory for sensitivity and cost-effectiveness. Nanodiagnostic not only aims to meet the demands of clinical diagnostic for increased sensitivity, but also pretends to achieve an early detection of the disease. The fact that most nanotechnology-based assays are perfectly adaptable to automation, and do not require a separation test, makes them diagnostic tools very appealing for scientists and clinicians (94).

Nanotechnology promises to play an important role in the future development of diagnostic and therapeutic methods. Whether nanodiagnostics will replace current diagnostic methods remains to be proved. Many aspects of these nanodiagnostics techniques need to be evaluated further, especially the safety issues (94, 95).

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